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THE EXPRESSION OF MGMT AND KU80 IN PRIMARY CENTRAL NERVOUS SYSTEM LYMPHOMA AND PROGNOSTIC SIGNIFICANCE

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The Expression of MGMT and Ku80 in Primary Central Nervous System Lymphoma and Prognostic Significance

1. Introduction

1.1 Epidemiology, pathological classification, prognostic immunophenotype and prognostic factors in primary central nervous system lymphoma (PCNSL)

1.11 Epidemiology of PCNSL

Primary central nervous system lymphoma (PCNSL），one of the uncommon extra nodal lymphomas, is defined as a lymphoma involving the brain, leptomeninges, eyes, or spinal cord without evidence of systemic disease. PCNSL usually is a diffuse large B-cell lymphoma (DLBCL) with a tropism for the CNS microenvironment. They are lacking lymphoid structures or aggregates. The incidence of PCNSL, in contrast, is now increasing although it was a rare diagnostic entity during the last 3 decades [1-4]. It has been reported in both immune compromised and immune competent patients, and it accounts for 1 to 3 per cent of all primary brain tumors [3-5]. The reported incidence is highest among patients with the acquired immunodeficiency syndrome(AIDS), (1.9 to 6%). PCNSL continues to be a prominent AIDS-defining illness [6].

Much attention has been given to the reported increase in incidence over the past 30 years, and there has been significant speculation as to the cause[7]. Some studies have shown stable or decreasing incidence rates and suggest that at least part of the reported increase may have been related to the acquired immunodeficiency syndrome epidemic [8]. The increasing incidence is not solely a result of its association with human immunodeficiency virus infection or other causes of acquired immunosuppressant condition such as transplantation. It also is not explained by improvement in diagnostic techniques.

1.12 Pathological classification of PCNSL

The diagnosis can be difficult, and repeated biopsies may be required once there is clinical suspicion of lymphoma. Brain biopsy remains the gold standard for

the diagnosis of PCNSL despite the fact that its symptoms and signs vary based on the anatomical localization and the extent of the lesion [9-11]. Tissue diagnosis is usually best obtained by means of stereotactic biopsy. The PCNSL shows a characteristic angiocentric pattern, forming cuffs of tumor cells within and around cerebral blood vessels. The tumor infiltrates the brain parenchyma with clusters or as individual cells. Most of the PCNSLs show a diffuse growth pattern with poorly defined margins, whereas a follicular growth pattern has not observed in PCNSL[5].

Starting from "perithelial sarcoma" named by Bailey to the descriptor and “reticulum-cell sarcoma–microgliomatosis”, the nomenclature for primary central nervous system lymphoma has undergone considerable evolution until the tumors were finally defined as “primary central nervous system lymphoma”. According to the Revised European-American Lymphoma (REAL) classification and the WHO classification in central nervous system tumors, the vast majority of PCNSL is classified as B-cell lymphomas; the most common histology is the diffuse large B-cell lymphoma(DLBCL), the majority of which are germinal center in origin [12]. Other common B-cell histologies include low-grade B-cell lymphoma, marginal zone B-cell lymphoma, plasmocytoma and intravascular B-cell lymphoma. T-cell variants are rare and account for less than 4% of all PCNSL in the western world.

1.13 Prognostic immunophenotype

Several recent studies have put emphasis on the analysis of protein expression of selected markers by IHC methods in patients with DLBCL, in order to define immunophenotypic profiles that better identify risk groups and prognostic assessments. It is met with general acceptance that Bcl-2, Survivin, Cyclin D, Ki67 and P53 are significantly prognostic biomarkers in non-CNS DLBCL [13]. Saez et al analyzed the expression of 52 proteins in DLBCL and generated a different model derived from logistic regression analysis and based on expression of eight markers (cyclin E, CDK1, CDK2, SKP2, EBER, MUM1, Rb-P, and BCL6) [14].

However, these biomarkers expressed in CNS DLBCL are not of the same prognostic significance as in non- CNS DLBCL. The tumor suppressor gene p53, cell

cycle regulatory molecules Ki67 and antiapoptotic protein bcl-2 in PCNSL are expressed in a similar fashion as in extracerebral B-cell lymphomas. However, they seem not to have prognostic implications [15]. Lin CH concluded the same for BCL-2 in PCNSL [16]. Although, Chang CC et al. assume a prognostic value for p53 expressions in immune competent patients with primary CNS DLBCL [17], there is no recent literature regarding this issue. Survivin expression has been associated with a significantly shorter 5-year survival in patients with DLBCL [18], but not in PCNSL [19].

Recently, several studies were focused on the identification of clinically relevant prognostic markers for PCNSL. Kunishio found p27 is a predictor of prognosis in patients with PCNSL [20]. D'Haene repoted that endothelial hyperplasia and/or endothelial galectin-3 expression was shown to be an independent prognostic factor for PCNSL patients treated with methotrexate-based chemotherapy [21]. These immunophenotypes in PCNSL on the other hand were seldom studied due to its low incidence. There is still uncertainty with regard to many proteins expressions in PCNSL.

1.14 Prognostic factors in PCNSL

Prognostic factors are important not only for discrimination of patients with PCNSL into specific risk groups and for the identification and assessment of appropriate therapies and predict survival, but also for comparing results of clinical trials. Since traditional prognostic markers in non-CNS DLBCL, such as staging and International Prognostic Index scores are not applicable to primary CNS DLBCL. Several studies were initiated to search for new markers predicting survival and selecting an adequate therapy.

Prognostic pathobiological biomarkers in PCNLSL have been described. Clinically, the International Extranodal Lymphoma Study Group (IELSG) has designed a score for PCNSL. This score was derived from a retrospective analysis of 378 patients from 48 centers [22]. Age older than 60 years, Eastern Cooperative Oncology Group performance status higher than 1 (KPS < 70), elevated serum LDH

level, high CSF protein concentration, and involvement of deep regions of the brain (periventricular regions, basal ganglia, brainstem, and/or cerebellum) are correlated with an negative prognosis. These 5 parameters comprise the elements of the IELSG prognostic score. Another score based purely on age and KPS, was developed at the Memorial Sloan-Kettering Cancer Center and externally validated by using data from prospective trials from the Radiation Therapy Oncology Group [23]. This model score is simple to use and depends on data that are collected in virtually all patients.

1.2 O6-Methylguanine DNA methyltransferase (MGMT)

1.21 MGMT protein molecular structure and function

In humans the MGMT gene is localized on chromosome 10q26 and contains five exons and four introns (length > 170 kb). The promoter region is CpG-rich, lacks TATA and CAAT boxes and has ten Sp1 transcription factor binding sites and two glucocorticoid response elements (GREs). MGMT protein, as a DNA repair protein, is a small enzyme-like substance of 207 amino acids (MW of 23 kDa). Most of our knowledge about MGMT is based on observations after exposure to alkylating agents [24-26]. In several ways, MGMT protects the cellular genome from the mutagenic effects of alkylating agents [25].

The MGMT-mediated repair process is unique and differs from other DNA repair pathways because MGMT is not part of a repair complex but acts alone. It restore the nucleotide to its native form by specifically removing the methyl group from the O6 position of guanine without causing any DNA strand breaks [25, 26]. This repair mechanism mediated by MGMT involves the transfer of the alkyl group from the alkylation site of the DNA to an internal active site represented by a cysteine (Cys) residue in the amino acidic sequence of the MGMT protein. After its interaction with O6-meG, the alkylated form of MGMT is no more available for DNA repair. For this reason this acceptor molecule cannot be considered an actual enzyme, and it is a so-called suicide enzyme. Because one molecule of MGMT removes one alkyl molecule, an excess of DNA adducts at the O6-position could completely deplete MGMT [27], therefore, it is an ideal target.

MGMT is ubiquitously expressed in normal human tissues, although at variable extent in selected tissues and on individual bases[28], but is overexpressed in all types of human tumors, including colon cancer, glioma, lung cancer, breast cancer, leukemia, lymphomas, and myeloma. The level of MGMT expression is protean in various tumor tissues because of epigenetic inactivation of the MGMT gene. In particular, hypermethylated CpG islands in the MGMT promoter seem to be the most important mechanism for MGMT gene silencing and for the down-regulation of the expression. Several studies have reported transcriptional silencing of this gene in up to 50% [29]. MGMT silencing is also often observed in tumors in which a number of other genes are suppressed by methylation. The silenced gene lists are not consistent in between tumors or tumor types[30]. Thus, the epigenetic alteration of MGMT behaves like a carcinogenic marker [27].

1.22 MGMT and chemosensitivity

How to predict and surmount the cell resistance in tumor chemotherapy is a complex problem. The mechanism of MTMG is to remove alkyl groups created by alkylating chemotherapy and therefore induces chemoresistances. It has been observed that MGMT gene expression seems to be related to the methylation of the MGMT promoter, MGMT enzyme activity, protein expression and cell resistance to anti-tumor alkylating agents by a series of recently experiments, which could predict a possible chemosensitivity. Therefore the study of MGMT status could be of therapeutic and prognostic interest.

Epigenetic silencing of MGMT expression by promoter methylation is a common event in human neoplasia. Methylation of the MGMT promoter region can also increase mutations in cancer[31]. It has been found that those tumors with high MGMT activity and abundance of MGMT protein were resistant to alkylating chemotherapeutics, while those with low MGMT activity and little MGMT protein expression were sensitive. Hence, MGMT behaves as a predictor of response to chemotherapy and also may be a prognostic biomarker. Given MGMT is one of the most important factors determining drug resistance to alkylation, strategies have been

developed to inhibit MGMT expression in tumors with the aid of MGMT inhibitors, and further enhance the anti-neoplastic efficiency of alkylating agents.

1.24 MGMT and brain tumors

Most of studies assessing the MGMT status in brain tumors focused on serial patients with glioma. The level of MGMT protein in malignant glioma varies widely ranging from almost undetectable to very high level. Esteller et al investigated the relationship between MGMT promoter methylation and response to carmustine in 47 patients with gliomas. In this study, MGMT gene promoter methylation was associated with a better response to chemotherapy, greater overall survival and longer time to progression [32]. Several studies reported similar results recently [33, 34]. The low MGMT protein expression was further identified as an independent favorable prognostic factor in terms of OS [35]. These results were consistent with the findings in the large phase III EORTC/NCIC trial conducted for patients with newly diagnosed GBM [36].

Only few studies about the MGMT expression in other brain tumors were sporadic described in the recent decade. Andersson and colleagues evaluated the immunohistochemical expressions and distribution of MGMT in low- and high-grade astrocytoma, oligodendroglioma and in different subgroups of meningioma. They revealed a marked heterogeneity in the expression and distribution which may be of importance in the selection of individualized chemotherapy [37]. De Robles et al found that none of the meningiomas showed MGMT gene promoter methylation and concluded that there is no biological rational to suggest that TMZ might have significant anti-meningioma activity [38]. Gonzalez-Gomez et al estimated the methylation status of multiple genes in Schwannomas, found a MGMT methylation of 20% with no significant correlations between the MGMT status and clinical features [39]. Lassaletta and co-workers confirmed the similar results [40]. Kovacs et al reported a TMZ treatment caused marked clinical improvement in a 46-year-old man with an aggressive and prolactin secreting pituitary tumor showing a low MGMT expression [41]. Widhalm et al suggested the MGMT expression may serve as an

additional prognostic factor in nonfunctioning pituitary adenomas [42]. The MGMT status in PCNSL and its correlation to clinical outcome is still unknown.

1.3 Ku80 introduction

1.31 Ku80 protein molecular structure and function

Ku80 (Ku86 in higher eukaryotes), a DNA repair protein, is derived from the XRCC5 gene localized to chromosome 2q33`q35 in human cells. Ku80 forms a heterodimer with Ku70, called Ku, which binds to DNA ends, nicks, gaps, and hairpins. In vitro, Ku forms a complex called DNA-dependent protein kinase (DNA-PK) by associating with a 450-kDa catalytic subunit and DNA-PKCS. Ku80, Ku70, DNA-PKCS, Xrcc4, and DNA ligase IV are critical for the repair of DNA ends by nonhomologous end joining (NHEJ). The Ku plays a key role in multiple nuclear processes, e.g., DNA repair, chromosome maintenance, transcription regulation and VJ recombination [43-45]. Although both Ku proteins and DNA-PKcs bind independently to the DNA ends, the greater part of this function is performed by the Ku70/Ku86 heterodimer, rather than DNA-PKCS itself [46].

Mice deleted for Ku70 or Ku80 exhibit hypersensitivity to γ -radiation, defective V(D)J recombination, genomic instability and early aging with low-cancer levels [47, 48]. This similar phenotype suggests Ku70 and Ku80 function is restricted to the Ku heterodimer. However, there is reason to believe Ku70 or Ku80 may function independent of the Ku heterodimer. Each subunit enters the nucleus through a different nuclear localization signal [49, 50] and Ku70 levels increase in response to γ-radiation without Ku80 [51]. Li et al suggested p53-mutant fibroblasts are more sensitive to streptonigrin and paraquat when deleted for Ku80 as compared with Ku70. Thus, Ku80 may function outside the Ku heterodimer to influence DNA damage repair [52].

1.32 Ku80 and radiosensitivity

DNA double-strand breaks (DSB) are the major lethal lesions induced by ionizing radiation. Cells mutated by the deletion of any of these genes are hypersensitive to ionizing radiation and defective in repairing DNA double-strand

breaks. The capability for DNA DSB repair is crucial for inherent radiosensitivity of tumor and normal cells. The success of DSB repair in tumor cells is the major cause for radiotherapy failure, leading to prolonged tumor cell survival. Thus, molecules that are involved in DSB repair may be potential prognostic markers for the prediction of radiotherapy outcome, and hence, for optimization of treatment.

Although there are exceptions, it is supported by results from several clinical studies that Ku protein expression is correlated with radiation treatment outcome [53-55] [56]. Upregulation of the Ku80 protein following ionizing radiation exposure has been reported previously [54, 57, 58]. Conversely, tumors with a low percentage of Ku80-positive cells tend to be radiosensitive.

1.33 Ku80 and lymphoma

There are some evidences that NHEJ mutations are lymphomagenic in humans. Artemis-deficient patients display aberrant chromosome rearrangements in peripheral blood lymphocytes and develop B cell lymphomas, without overt immunodeficiency [59]. Badle et al. described a case with a hypomorphic mutation in ligase IV, which is associated with acute lymphoblastic leukemia and radiosensitivity [60]. Inherited or acquired mutations in NHEJ factors could be important risk factors for developing lymphoid neoplasms.

Despite many studies performed with established cell lines, little is known about Ku80 expression in lymphoma. Some studies were performed in patients with chronic lymphocytic leukemia (CLL) [61] and myeloma [62]. Chen TY and coworker stated the amount of Ku80 expression in ALL was moderately correlated with peripheral white blood cell counts, and high Ku80 expressers tended to respond poorly to therapy. They suggested that Ku80 might contribute to generally poor prognoses in adult ALL [63]. So far the expression and significance of Ku80 in primary central nervous system lymphoma or DLBCL has not been analyzed.

1.4 Questions and aims of the study

As mentioned above, PCNSL，as one of the uncommon extranodal lymphomas, has been recently paid more attention especially for its increasing incidence, unsatisfactory therapy and poor prognosis. Prognostic factors are important not only for division of patients with PCNSL into specific risk groups for identification and assessment of appropriate therapies and to predict the survival, but also for comparing results of clinical trials. It is clear that the biological mechanism involved in pathogenesis of PCNSL are complex but deserve further study. Obviously, a better insight of its biology is crucial to improve the prognosis. If it were possible to interfere with the chemosensitivity and radiosensitivity of PCNSL, an alternative therapeutic approach might be found.

Given MGMT is one of the most important factors determining drug resistance while Ku80 determining radiosensitivity, the expression of MGMT and Ku80 in PCNSL remains unclear. Herein, we postulate that the expression of the Ku80 and MGMT in PCNSL is of interest for the therapy. It is for this reason that our study was designed to detect the expression of MGMT and Ku80 on PCNSL by IHC staining to address the following questions: 1) to determine the expression level of Ku80 and MGMT for correlation with the clinical status of the patients. And 2) to evaluate the relationship between Ku80, MGMT expression level and clinical outcomes, thus determine whether these immunophenotypes were prognostic factors in PCNSL. They may be new markers for anticipating curative effects. These also might be strategy to the genes for future therapy in primary central nervous system lymphoma.

2. MATERIALS AND METHODS

2.1 Patients and tissue samples

2.11 Patient eligibility

PCNSL tissues and clinical data were collected from 49 patients who were diagnosed and treated at the Department of Neurosurgery, UK S-H Campus Kiel, Germany. Patients were eligible for this study if they had been diagnosed with PCNSL. Patients selected criteria for this study: (1) Histological characteristics of these cases fulfilled the criteria of the World Health Organization criteria of lymphoid neoplasms for PCNSL, (2) Patients were required to have a life expectancy greater than 1 months, with detailed clinical data at diagnosis and therapy and during follow up, (3) Availability of adequate tissue specimens for histologic typing and immunohistochemistry, (4) Have adequate hematologic, renal and hepatic function. Patients who had a history of HIV infection or who were receiving immunosuppressive therapy were excluded from the study. Patients previously treated by radiotherapy or chemotherapy were excluded. Patients who died from recent complications postoperatively weren't involved in the study. The study also excluded lymphomas located in spinal canal.

In order to compare expressions of Ku80, MGMT in primary cerebral lymphoma and secondary cerebral lymphoma, 6 secondary cerebral lymphomas were involved in this study.

2.12 Clinical data of patients

Between May 1994 and Jan 2009, there were a total of 122 patients with a new histological diagnosis of PCNSL by the World Health Organization (WHO) criteria in the Department of Neurosurgery, UK S-H Campus Kiel, Germany. Among them, 49 patients were enrolled in this study. Clinical follow-up was obtained until July 15, 2009, or until death or lost follow-up. 20 patients had undergone craniotomy at the time of initial diagnosis and 29 patients had undergone stereotactic biopsy. Differential indication for open craniotomy was seen when the preoperative diagnosis of a space-occupying intracerebral lesions based on MRI scans was not suggestive of

primary cerebral lymphoma. Once PCNSL was diagnosed and at least two weeks had gone by postoperatively for physical recovery, chemotherapy or/and radiotherapy was started in the respective depts. of medical oncology or radiotherapy. Treatment of patients varied, depending on individual conditions, the stage of their disease, date of diagnosis, institution, and healthy conditions. All patients were followed up postoperatively by clinical examination and MRI scanning at three month intervals or when recurrence was suspected. Those patients who got a complete remission were followed up by phone calls and were not regularly reexamined if being in good health.

2.13 Histological classification

Histological characteristics of 49 PCNSLs were DLBCL. 2 cases fulfilled the criteria of the intravascular subtype for DLBCL. The 6 secondary cerebral lymphomas were classified as DLBCL.

2.2 Immunohistochemistry

2.21 Experimental instruments

Medical pressure cooker：Sicomatic-L, Germany

Electronic precision scale：A200S-*DI，SARTORIUS GMBH GOETTINGEN, Germany

Micropipetters and tips：EPPENDORF，Germany

Optical microscopy：OLYMPUS，BH-2，Japanese

2.22 Experimental reagents

（1）TBS （Tris-GerufferK Kodsalzlsg pH7,0）

Tri-sodium base	(Sigma T 1503)	0.9 g
Tris-HCL	(Sigma T 3253)	6.85g
NaCL	(Merck 1.o64o4)	8.78g
Distilled water		1000 ml

（2） EDTA-Puffer (TEC-Puffer pH7.8)

Tri-sodium base	(Sigma T 1503)	2.5 g
EDTA	(Merck 1.08418)	5.0 g
Tri-Sodium Citrate	(Merck 1.06448)	3.2g

Distilled water 1000 ml

（3）AEC Reagent：Sigma

（4）Ku80 Mouse Monoclonal Antibody: 1:200 LAB VISION

（5）MGMT Mouse Monoclonal Antibody: 1:60 Thermo

（6）Second antibody: Anti-Mouse and Rabbit Histofine:NICHIREI BIOSCIENCEC INC, Germany

（7）Tris Merck Germany

（8）HCL Merck Germany

（9）NaCL Merck Germany

（10）Non-Fat Dry Milk Roth Germany

（11）Hematoxylin Solution Merck Germany

（12）3% hydrogen peroxide Merck Germany

2.23 Control samples：

According to antibody data sheets, normal tonsil tissues obtained are served as normal control samples for Ku80, and colon cancer tissues are for MGMT. For positive and negative (omission of first antibody) control of the staining reaction, these sections were stained in parallel to all cases in our study cohort. Each antigen has a preferred method of antigen retrieval, and each antibody was optimal diluted. Positive control experiments are performed to find optimal staining conditions before immunohistochemical stains can be proceeded.

We tried staining without antigen retrieval, and also used the heat-induced epitope retrieval with a pressure cooker. Antigen retrieval was tested in Tris/EDTA pH 6.0, 7.8 and 9.0 buffers. The antigen retrieval time was controlled for 3 minutes (Ku80) and 10 minutes (MGMT) as soon as the cooker had reached full pressure. Antibody concentrations were diluted to 1:20, 1:40, 1:60, 1:80 for MGMT, and to 1:100, 1:200 for Ku80, as recommend on the data sheets. Finally a dilution of 1:60 was determined as the optimal dilution for MGMT, and 1:200 for Ku80.

2.24 Experimental process

The basic steps of the IHC protocol are as follows: fixing and embedding the tissue, cutting and mounting the section, deparaffinizing and rehydrating the section, Antigen retrieval, Immunohistochemical staining, counterstaining (if desired), dehydrating and stabilizing with mounting medium, viewing the staining under the microscope

2.241 Deparaffinizing and rehydrating the sections

Formalin-fixed, paraffin-embedded sections (3-µm thick) were dewaxed in xylene and rehydrated by passage through a graded ethanol series to distilled water. The step in details is performing the following washes in proper order with sections placed in a rack.

1). Xylene:	2 x 5 minutes
2). Xylene 1:1 with 100% ethanol:	2 x 5 minutes
3). 100% ethanol:	2 x 2 minutes
4). 96% ethanol:	2 x 2 minutes
5). 70 % ethanol:	2 minutes
6). Running cold distilled water to rinse	

2.242 Antigen retrieval

The appropriate antigen retrieval buffer is added into the pressure cooker and the slides transferred from the distilled water to the pressure cooker. The pressure cooker is placed on the hotplate and turned on full power after securing the lid of the pressure cooker. The cooker has reached full pressure in 3 minutes (10minutes for MGMT antigen retrieval). The hotplate is turned off and placed in an empty sink. The pressure release valve is activated. Once de-pressurized, the slides are rinsed with distilled water.

2.243 Immunohistochemical staining process

1). Slides are kept in distilled water for 1 minute.

2). Slides are kept in 70% ethanol for 1 minute.

3). 96% ethanol for 1 minute.

4). Endogenous peroxidases were blocked with 3% hydrogen peroxide in Tris-buffered saline (TBS) for 10 minutes.

5). 96% ethanol for 1 minute.

6). 70% ethanol for 1 minute.

7). Distilled water for 1 minute.

8). Blocking of nonspecific binding was accomplished in 5% skim milk for 10 minutes. (This step is only used for MGMT)

9). Slides were washed in distilled water for 1 minute. (This step is only used for MGMT)

10). Sections were washed 3 times in TBS for 2 minutes

11). Drain slides for a few seconds (do not rinse) and wipe around the sections with tissue paper

12). Prepare the Primary antibody diluted (1:60 for MGMT and 1:200 for Ku80) according to the manufacture's protocol. 100 µL of primary antibody fine-tuned was added.

13). Anti-Ku80 mouse monoclonal antibody is raised in room temperature for 30 minutes, and anti-MGMT is raised in room temperature overnight (18 hours).

14). Slides were washed in distilled water for 2 minute after incubation.

15). Sections were washed 3 times in TBS for 2 minutes.

16). Incubated at room temperature for 30 minutes with 100 µL of anti-Mouse and rabbit histofine. (Secondary antibody）

17). Sections were washed 3 times in distilled water for 5 minutes.

18). Wash the slides 2 minutes in TBS for3 times.

19). Incubated for 20 minutes at room temperature with 100 µL AEC complex and 1 µL 3% hydrogen peroxide, and then washed in distilled water for 1 minute

20). Sections were counterstained for 5 minutes with hematoxylin.

21). Wash slides 5 minutes and then wash in distilled water for several

seconds, mounted using a drop of aqua tex.

2.25 Results evaluation

A neuropathologist without prior knowledge of the patients' clinical outcomes investigated all histologic specimens. Each tumor was evaluated for these gene proteins and given the percentage of positive cells. One thousand neoplastic cells per specimen were evaluated at x400 magnification and the ratio (%) of Ku80, MGMT immunoreactive neoplastic cells was counted. On the basis of the percentages of positive cells in the tumors, these tumors were defined as low Ku80 expression or low MGMT expression when there are no or fewer than 50% positive cells, and high expression when positive rate was equal to or more than 50%.

2.3 Statistical analysis

Complete remission (CR) was defined as the disappearance of all contrast enhancements in MRI in the absence of corticosteroids. Partial remission (PR) was defined as a ≥50% reduction in tumor size compared with the baseline MRI. End points of the study were OS and progression-free survival (PFS). PFS was evaluated from the first day of treatment to relapse, progression or death, or to the last date of follow-up, and overall survival (OS) was calculated from the first day of treatment of the tumor to death for any reason or to the last date of follow-up. Patients who did not experience the event of interest with respect to OS or PFS were considered as censored observations with time from first diagnosis to last follow-up visit as the censoring time.

A descriptive study of all the variables included in the study was carried out. The quantitative variables were expressed in terms of their centralization and dispersion measurements, and in some cases were categorized in accordance with their median value. Expressions of Ku80 and MGMT between subgroups were analyzed by One-way ANOVA method. Chi-square test was applied to estimate the relation between the expression of Ku80, MGMT and patient characteristics. Correlation analysis was performed between the expression of Ku80 and MGMT. Kaplan–Meier methodology was applied in order to determine the effect of the

different variables on survival. Parameters possibly correlated with disease progression and survival were age, gender, KPS at relapse, tumor localization, surgical procedure, and use of alkylating agents, radiotherapy, expressions of MGMT and ku80 protein. These variables were estimated with their mean and 95% confidence interval. The end-point variable of interest was overall survival. The log-rank test was applied in order to verify the probabilities of accumulated survival in accordance with different strata of variables. A $P<0.05$ value was considered to be of statistical significance. Analyses were performed with the use of SPSS 17.0.

3. RESULTS

3.1 Patient characteristics and treatment

49 patients with PCNSL and 6 patients with secondary CNS lymphoma were included in this retrospective study. All data of these patients are presented in Table 1.

Tab 1. Primary clinical data of the patient characteristics

No.	Sex	Age	Location	treatment	operation	Radio-therapy	Alkylating agents	Ku80 (%)	MGMT (%)	PFS (mon)	OS (mon)	S/D
1	M	62	CA	CT	craniotomy	no	yes	93.8	85.6	102.0	102.0	S
2	F	56	ML	RT	craniotomy	yes	no	46.5	44.3	5.5	7.5	D
3	F	76	CA	RT	biopsy	yes	no	31.4	8.5	6.0	6.0	S
4	M	57	CA	RT	craniotomy	yes	no	37.4	25.4	90.0	93.5	D
5	F	58	SL	RC	biopsy	yes	yes	22.8	64.6	12.0	12.5	D
6	M	79	SL	NT	craniotomy	no	no	84.4	59.4	0.0	1.0	D
7	F	64	ML	CT	biopsy	no	yes	94.8	84.2	0.0	1.0	D
8	F	77	SL	RC	biopsy	yes	no	7.4	5.6	4.0	4.0	D
9	M	66	CA	CT	biopsy	no	yes	94.4	87.2	0.0	1.5	D
10	F	80	SL	RC	biopsy	yes	no	73.4	73.0	4.0	4.0	D
11	M	73	CA	RC	biopsy	yes	no	80.4	9.4	1.5	5.0	D
12	M	52	OL	RC	biopsy	yes	yes	20.8	43.4	82.0	82.0	S
13	M	73	ML	CT	biopsy	no	yes	95.8	76.2	12.0	14.0	D
14	F	81	OL	RC	biopsy	yes	yes	59.6	78.2	2.0	2.0	S
15	F	45	ML	CT	biopsy	no	yes	90.9	80.5	0.0	2.0	D
16	M	56	SL	CT	craniotomy	no	yes	25.2	83.2	64.0	64.0	S
17	F	74	ML	NT	craniotomy	no	no	63.0	71.2	0.0	1.0	D
18	F	44	CA	RC	biopsy	yes	yes	44.0	77.6	60.0	60.0	S
19	M	77	SL	RC	biopsy	yes	yes	78.2	73.4	30.0	30.0	S
20	M	66	SL	CT	craniotomy	no	yes	84.2	76.4	1.0	4.0	D
21	M	85	ML	NT	biopsy	no	no	55.8	43.8	0.0	3.0	D
22	F	52	ML	CT	biopsy	no	yes	72.5	26.2	47.0	47.0	S
23	M	63	ML	RC	biopsy	yes	yes	72.6	89.2	9.0	12.0	D
24	M	44	CA	RC	craniotomy	yes	yes	48.2	62.4	27.0	27.0	S
25	F	59	SL	CT	biopsy	no	yes	21.4	5.4	30.0	30.0	S
26	M	68	ML	CT	craniotomy	no	yes	81.4	8.4	16.0	23.0	D
27	F	63	SL	RC	biopsy	yes	yes	63.4	31.4	6.0	15.0	D
28	F	46	SL	RC	biopsy	yes	yes	71.8	6.2	13.0	13.0	S
29	M	62	SL	CT	biopsy	no	yes	83.6	11.8	9.0	11.0	D
30	F	84	SL	RC	craniotomy	yes	no	85.6	8.4	0.0	2.5	D
31	F	77	CA	CT	biopsy	no	yes	67.4	53.6	13.0	13.0	S
32	F	78	SL	NT	biopsy	no	no	79.4	17.6	0.0	3.0	D
33	M	70	SL	RT	biopsy	yes	no	68.2	90.8	11.0	11.0	S

34	M	67	ML	RT	biopsy	yes	no	90.6	61.4	10.0	10.0	S
35	F	78	CA	NT	biopsy	no	no	72.4	74.8	2.0	3.0	D
36	M	59	CA	RC	biopsy	yes	yes	44.6	45.3	72.5	72.5	S
37	M	85	ML	RT	craniotomy	yes	no	86.2	26.5	0.0	3.0	D
38	F	67	ML	CT	craniotomy	no	no	67.4	94.4	0.0	1.0	D
39	M	58	CA	RC	craniotomy	yes	no	86.2	87.4	7.5	7.5	S
40	F	42	SL	CT	craniotomy	no	yes	95.6	89.4	9.0	9.0	S
41	M	57	SL	RC	biopsy	yes	yes	29.7	41.8	85.0	85.0	S
42	M	47	SL	CT	biopsy	no	yes	38.1	35.8	82.0	82.0	S
43	M	68	ML	RC	biopsy	yes	yes	75.2	48.6	89.0	89.0	S
44	F	73	OL	RT	craniotomy	yes	no	61.8	52.5	7.0	7.0	S
45	M	82	OL	RC	craniotomy	yes	no	65.0	11.8	145.0	145.0	S
46	M	71	OL	RC	craniotomy	yes	no	94.2	68.3	48.0	48.0	S
47	M	48	OL	CT	craniotomy	no	yes	70.6	23.2	40.0	40.0	S
48	F	75	OL	NT	craniotomy	no	no	21.4	13.2	16.0	18.5	D
49	F	6	OL	CT	craniotomy	no	yes	39.6	78.3	122.0	122.0	S
50	M	65	OL	RC	craniotomy	yes	yes	48.5	52.2	88.0	88.0	S
51	F	77	ML	CT	biopsy	yes	yes	64.8	93.2	2.0	11.0	D
52	F	67	ML	RC	biopsy	yes	UNK	9.2	23.4	35.0	36.0	D
53	F	71	SL	RT	biopsy	yes	no	67.5	33.4	2.0	4.5	D
54	F	60	CA	RC	craniotomy	yes	UNK	77.4	79.6	40.0	49.0	D
55	M	71	ML	CT	biopsy	no	yes	85.4	82.6	0.0	4.0	D

Abbreviations : OS, overall survival; PFS, progress free survival; M, male; F, female; S, survival; D, death; SL, single lobe; CA, central area; ML, Multiple lobes; OL, other location; CT, chemotherapy; RT, radiotherapy; RC, radiochemotherapy; NT , no treatment; Unk, unknown. No.50~55 patients were diagnosed as secondary CNS lymphomas.

This group comprised 26 men and 23 women, male: female ratio is 1.13, age varied from 6 to 85 years (mean 64.29 years). The lymphomas were located as follows: In 11 patients the tumor was located in central area such as cerebellar, brain stem thalamus, hypothalamus, para- or intra-ventricular, in 15 cases the tumor was restricted to a single lobe such as frontal, temporal, parietal, occipital lobe, more than one cerebral lobe was affected in 13 patients, in 8 patients lymphomas were located in the eye socket or spine.

All patients were diagnosed as PCNSL by pathologist according to the World Health Organization (WHO) criteria. Biopsies were taken in 20 patients via a craniotomy and in 29 patients via a stereotactic biopsy. Subsequent treatment of

patients varied, depending on individual conditions, the stage of their disease, date of diagnosis, institution, and health conditions As mentioned before,these patients were treated in the appropriate hemato-oncological departments. Based on these factors, 17 patients accepted HD-MTX-based, multi-agent chemotherapy and 7 patients were treated with sole radiotherapy. 19 patients with PCNSL underwent both chemotherapy and radiotherapy whereas no further therapy was applied in 6 patients (Tab. 2).

Tab. 2 Treatment protocols in 49 patients

Treatment	Radiotherapy	No radiotherapy	total
Chemotherapy	19（38.8%）	17（34.7%）	36
No chemotherapy	7（14.3%）	6（12.2%）	13
Total	26	23	49

36 patients were treated with polychemotherapy: CHOP +MTX protocol was used in 17 patients and B-ALL protocol was chosen in 11 patients. 8 patients accepted NOVEP or other protocol chemotherapy. A chemotherapy cycle of the CHOP+MTX protocol consisted of high-dose MTX, cyclophosphamide, doxorubicin, vincristine, and prednisone. Chemotherapy cycles were repeated every 21 days; a maximum of 6 cycles was planned. A chemotherapy cycle of the modified B-ALL protocol consisting of six alternating cycles (3 x cycles A, 3 x cycle B) of polychemotherapy after a prephase treatment of cyclophosphamide (CP) and prednisone. During cycle A, VM26, ifosphamide (IFO), methotrexate (MTX), cytarabine (Ara-C), vincristine, and dexamethasone were given. During cycle B, Ara-C, VM26 and IFO were replaced by doxorubicine and cyclophosphamide. As NOVEP didn't include any alkylating agents, these cases were of minor relevance concerning the MGMT status. In total 49 patients, 28 patients were included into protocol of chemotherapy including alkylating agents, 21 cases were treated without any alkylating agents. 25 patients underwent hyper-fractionated whole-brain radiotherapy (WBRT) with 40-50 Gy (2 Gy/day), which is considered equivalent to conventional irradiation with 20 fractions of 2 Gy, total 40

Gy. Among them, an additional 6-10 Gy boost to gross was performed. 24 patients had no radiotherapy. All patients were followed up. The time of follow-up varied from 1 month to 145 months (Mean 29.6 months). It was terminated until to death for any reason or to the last date of follow-up. The mean overall survival (OS) was 29.6 months (Range 1 - 145 months), and the mean PFS was 28.4 months (Range 0 - 145 months).

3.2 Ku80 expression in PCNSL

The Ku80 expression was determined by Ku80 immunohistochemistry. The absolute expression level of Ku80 in all groups classified according to variables were analyzed by One-way ANOVA method and listed in Table 3. It is found that a considerable variability in Ku80 expression level ranged between 7.4% and 95.8% in PCNSL, mean 64.1%. The results of Ku80 expression by age, gender, tumor location are presented in Tab. 4. 15 tumor samples (30.4%) demonstrated low Ku80 staining (positive rate < 50%，mean 31.9%±11.9%) (Fig. 1, 2), and 34 (69.6%) tumor samples demonstrated high Ku80 expression respectively (Positive rate >50%， mean 78.2%± 11.8%), (Fig. 3, 4).

The Ku80 expression level in PCNSL (N = 49) was not correlated with the gender of the patients. A significant difference in Ku80 expression (weak or high) could be found between age<65 years group and age≥65 years group (P=0.006), however, no statistical relevant correlation was found between age of patient and Ku80 expression level (P=0.136). This revealed that the Ku80 level in age≥65 years patients is usually higher than in patients whose age is less than 65 years. There was no significant difference in Ku80 expression between primary and secondary CNS lymphomas (P=1.000) or between different locations of tumors (P=0.139).

Tab. 3 Expressions of Ku80 and MGMT analyzed by One-way ANOVA method.

Variables	MGMT(mean±SD)	MGMT 95% CI	*p* value
Overall	51.3±29.5	42.8-59.8	
Sex(n)			
M (n=26)	52.9±27.8	41.7-64.1	
F (n=23)	49.5±31.9	35.7-63.3	0.692
Age			
< 65 (n=23)	53.1±29.1	40.6-65.7	
≥ 65 (n=26)	49.7±30.4	37.4-62.0	0.689
Tumor location			
SL (n=17)	45.5±32.4	28.8-62.2	
CA (n=11)	56.1±30.3	35.8-76.5	
ML (n=13)	58.1±27.3	41.6-74.6	
Others(n=8)	46.1±27.8	2.9-69.3	0.614
Surgical approach			
Biopsy(n=29)	49.8±29.3	38.7-61.0	
Craniotomy(n=20)	53.5±30.6	39.2-67.8	0.676
Treatment			
CT(n=17)	58.8±32.5	42.1-75.5	
RT(n=7)	44.2±27.3	18.9-69.5	
RC(n=19)	48.7±29.3	34.6-62.9	
No(n=6)	46.7±26.6	18.8-74.5	0.630
Chemotherapy			
Yes(n=36)	53.5±30.8	43.1-63.9	
No(n=13)	45.3±25.9	29.7-61.0	0.399
Alkylating agents			
Yes(n=28)	56.0±28.3	45.0-67.0	
No(n=21)	45.1±29.6	31.2-59.1	0.207
Radiotherapy			
Yes(n=26)	47.5±28.3	36.1-59.0	
No(n=23)	55.6±31.0	42.3-69.0	0.342
yes	55.2±23.5	40.2-70.1	
no	50.1±31.5	39.6-60.6	0.609
Ku80 expression			
Low(n=15)	42.3±26.9	27.4-57.2	
High(n=34)	55.3±30.2	44.8-65.8	0.159
MGMT expression			
Low(n=23)	23.6±15.3	16.9-30.1	
High(n=26)	75.9±11.6	71.2-80.5	

Abbreviations : M, male; F, female; SL, single lobe; CA, central area; ML, Multiple lobes; CT, chemotherapy; RT, radiotherapy; RC, radiochemotherapy; CI, Confidence Interval; SD, Std. Deviation.

Baseline patient characteristics and Ku80 expression

Study, n (%)	Ku80 (low)	Ku80 (high)	p value
Sex			
Male	7 (14.3%)	19 (38.8%)	
Female	8 (16.3%)	15 (30.6%)	0.551
Age			
< 65 years	12 (24.5%)	11 (22.4%)	
≥ 65 years	3 (6.1%)	23 (46.9%)	0.006
Tumor location			
Single lobe	6 (12.2%)	11 (22.4%)	
Multiple lobes	1 (2.0%)	12 (24.5%)	
Central area	5 (10.2%)	6 (12.2%)	
others	3 (6.1%)	5 (10.2%)	0.139
Diagnosis			
Primary CNSL	15 (27.3%)	34 (61.8%)	
Secondary CNSL	2 (3.6%)	4 (7.3%)	1.000

3.3 **MGMT expression in PCNSL**

The level of MGMT expression was determined by MGMT immunohistochemistry. There is a considerable variability in MGMT expression level ranging between 5.4% and 94.4% in PCNSL (Mean 51.3%). The results of MGMT expression by age, gender, tumor location are presented in Tab. 5. 23 tumor samples (46.9%) demonstrated low MGMT staining (Positive rate < 50%, mean 23.6%± 15.3%), (Fig. 5, 6), and 26 (53.1%) tumor samples demonstrated intermediate and high MGMT expression respectively (positive rate >= 50%, mean 75.9%±11.6%), (Fig. 7, 8). The MGMT expression level in PCNSL (N = 49) was neither correlated with age of the patient nor with gender. There was no significant difference in MGMT expression between different locations of tumors (P=0.856). Differences in MGMT expression between primary and secondary CNS lymphomas did not reach significance (P=0.844).

Baseline patient characteristics and MGMT expression

Study, n (%)	MGMT (weak)	MGMT (strong)	p value
Sex			
Male	13 (26.5%)	13 (26.5%)	
Female	10 (20.4%)	13 (26.5%)	0.648
Age			
< 65 years	12 (24.5%)	11 (22.4%)	
≥ 65 years	11 (22.4%)	15 (30.6%)	0.490
Tumor location			
Single lobe	9 (18.4%)	8 (16.3%)	
Multiple lobes	6 (12.2%)	7 (14.3%)	
Central area	4 (8.2%)	7 (14.3%)	
others	4 (8.2%)	4 (8.2%)	0.856
Diagnosis			
Primary CNSL	23 (41.8%)	26 (47.3%)	
Secondary CNSL	2 (3.6%)	4 (7.3%)	0.844

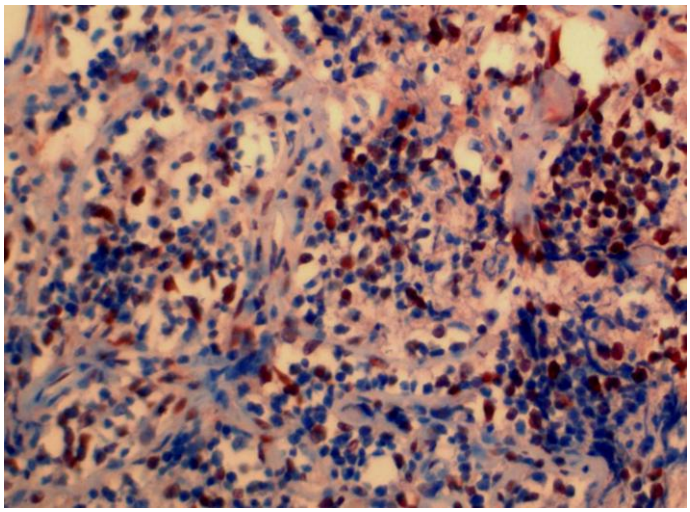

Fig. 1 Ku80 staining ×200

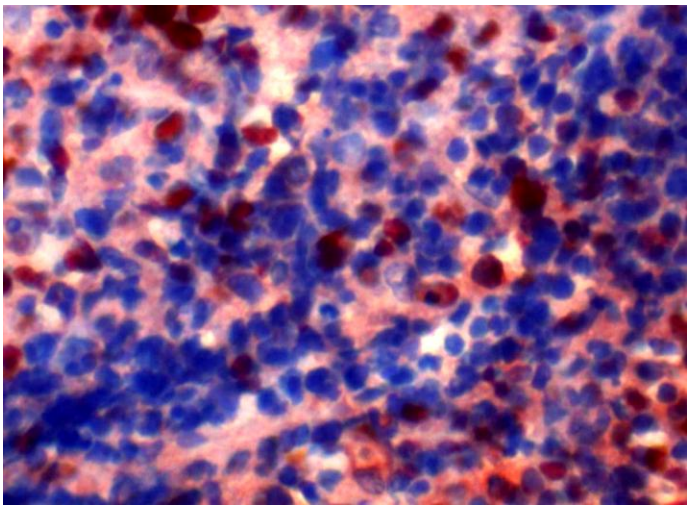

Fig . 2 Ku80 staining ×400

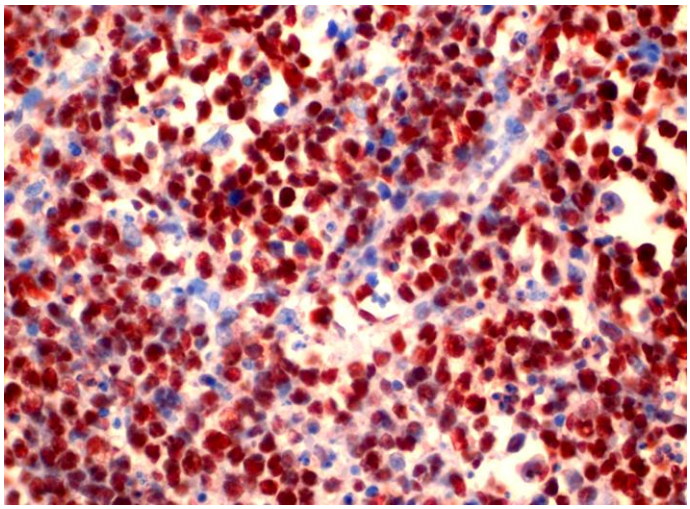

Fig. 3 Ku80 staining ×200

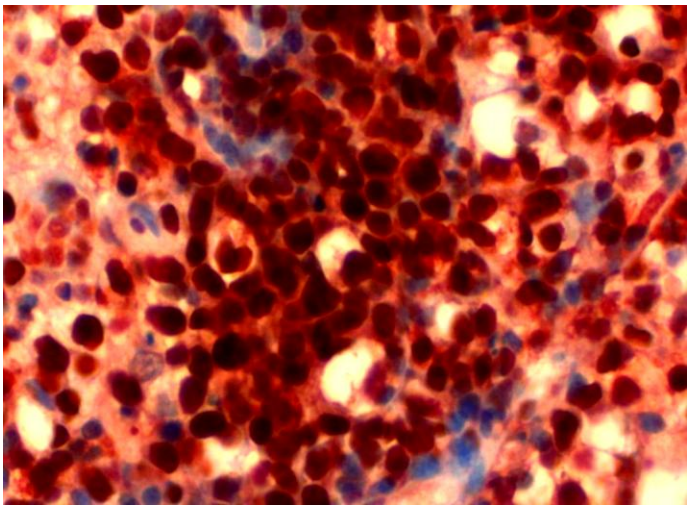

Fig. 4 Ku80 staining ×400

Fig. 1~4 Representative photomicrographs showing immunostaining for Ku80 in PCNSL samples. Fig. 1, 2 Immunohistochemical staining demonstrates the low expression of Ku80 in the PCNSL tissue, and strong nuclear staining could be observed in Fig. 3, 4.

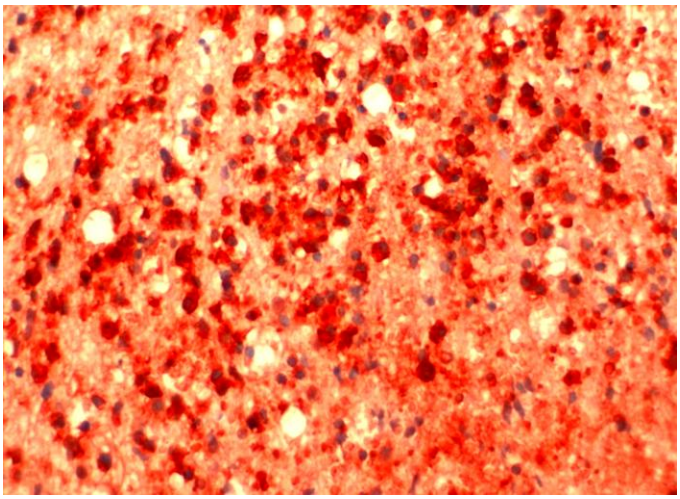

Fig. 5 MGMT staining ×200

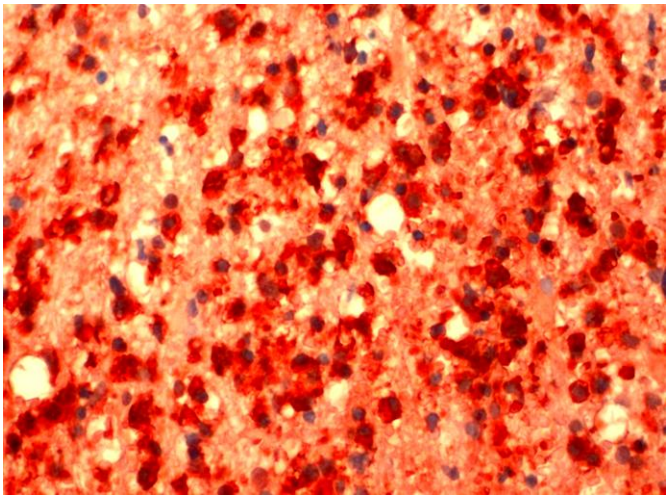

Fig. 6 MGMT staining ×400

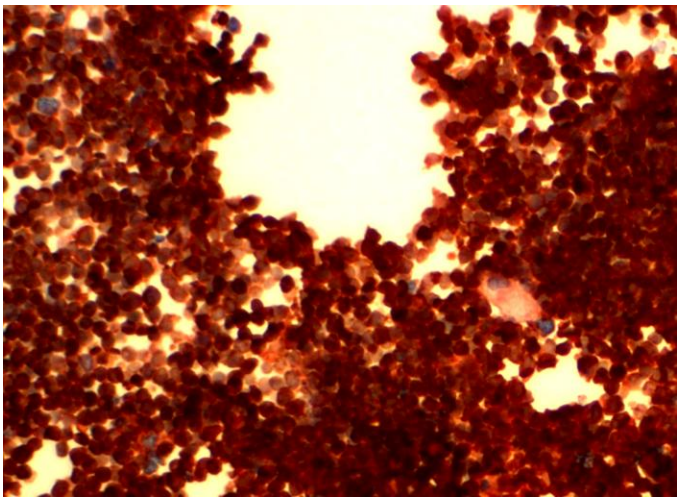

Fig. 7 MGMT staining ×200

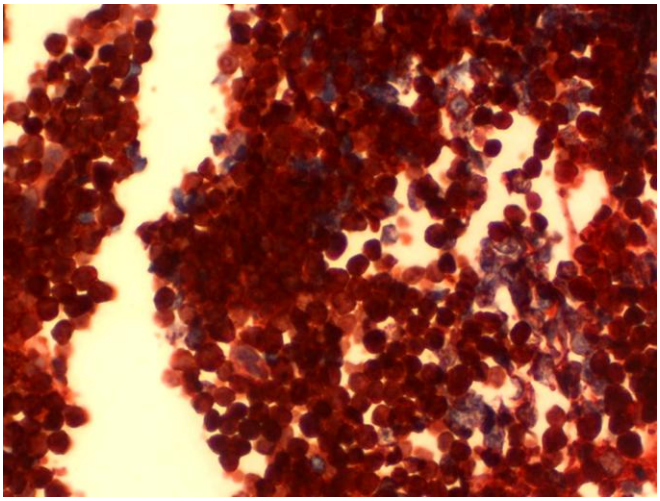

Fig. 8 MGMT staining ×400

Fig. 5~8 Representative photomicrographs showing immunostaining for MGMT in PCNSL samples. Fig 5, 6 Immunohistochemical staining demonstrates the media expression of MGMT in the PCNSL tissue, and Fig 7, 8 demonstrates strong cytoplasmic staining.

3.4 Relevance analysis between Ku80 expression and MGMT expression

Strong positivity of MGMT staining was observed in 23 (46.9%) of 49 PCNSL cases and strong Ku80 expression was found in 15 (30.4%) patients. Among the 49 cases that were interpretable for both proteins, 21 cases (42.9%) were positive for both Ku80 and MGMT, 5 cases (10.2%) were positive only for MGMT, 13 cases (26.5%) were positive only for Ku80, and 10 cases (20.4%) were negative for both proteins (Tab 6). A strong trend was found toward poor prognosis in MGMT/Ku80 positive as compared with MGMT/Ku80 negative cases. The difference, however, did not reach statistical significance by Kaplan-Meier analysis (P = 0.244), (Tab. 7.). No significant difference was observed between levels in Ku80 expression and MGMT expression (P=0.066). However, a correlation was found between the expression of these two proteins (r=0.311, P=0.029). Co- expression of Ku80 and MGMT existed in patients with PCNSL.

Correlation between Ku80 expression and MGMT expression

Location Protein	Ku80(low)	Ku80(high)	Total
MGMT(low)	10(20.4%)	13(26.5%)	23
MGMT (high)	5(10.2%)	21(42.9%)	26
Total	15	34	49

3.5 Survival analysis in PCNSL

The Kaplan-Meier method was employed to calculate the progression-free survival and overall survival rates. The significance of the difference in the survival curves was calculated with the log-rank tests. Kaplan-Meier survival curves of patients were analyzed according to tumor location, surgical approach, treatment protocol, alkylating agents, and the expression of MGMT and Ku80. Tumor locations were divided into four groups stated as above: central area (such brain stem, thalamus, hypothalamus, and para- or intra-ventricular), a single cerebral lobe (such as frontal, temporal, parietal, occipital lobe), multiple cerebral lobes and other location (such as

eye sockets or spinal). The treatment protocol was categorized into four groups described as above: chemotherapy, radiotherapy, radiochemotherapy and no chemo- or/and radiotherapy. These patients were also classified into alkylating agents group and non- alkylating agents group. The surgical approach was categorized into (stereotactic) biopsy and craniotomy. The expression of MGMT and Ku80 was evaluated by immunohistochemical staining, Those patients were divided into a weak expression group (Positive rate < 50%) and high expression group (Positive rate >= 50%), there are comparable between these subgroups without statistical differences on the expression level of Ku80 and MGMT (Tab 3, P>0.05).

Kaplan-Meier analysis revealed that patients who had high Ku80 expression had significantly shorter median survival time (MST) than patients who had low Ku80 expression (55.3 months vs. 80.4 months; P =0.036; log-rank test) (Fig. 9). Different results were obtained for patients who had high and low MGMT expression (MST:59.7 months vs.63.9 months); but this difference did not reach statistical significance (p 0.706, long-rank test). Similarly, no difference was seenbetween patients who had undergone biopsy vs. patients who had undergone craniotomy (p 0.796, log-rank test), Tumor location in central brain areas, as expected, was correlated with a significantly higher risk of death (P =0.014) (Fig. 10). Similar results were obtained for patients without any therapy compared to patients who underwent chemotherapy or/and radiotherapy (P =0.002) (Fig.11).Radiotherapy alone was not a significant prognostic factor for survival. Patients older than 65 years, as expected, were found to have a significantly higher risk of death (45.3 months vs. 78.6 months; P =0.011; log-rank test).

Kaplan-Meier analysis also discovered, that patients who underwent chemotherapy with alkylating agents had significantly longer MST than patients who didn’t (78.1 months vs. 41.2 months; P =0.007; log-rank test) (Fig.12). In addition to these, patients who underwent both alkylating agents and radiotherapy had significantly longer MST than patients who didn’t. However, it did not reach

statistical significance (67.5 months vs. 57.8 months; P =0.059; log-rank test). These findings supported the conclusion that alkylating agents play an important role in treatment of patients with PCNSL. In Tab.7, it is shown that patients' age, tumor location, treatment protocol, alkylating agents and Ku80 were related with prognosis (OS) in PCNSL. Kaplan-Meier analysis between variables with PFS revealed the same statistical results as OS (Tab.8).

Tab. 7 Kaplan–Meier analyses for the correlation between variables and overall survival

Variables	OS Mean	95% CI	log-rank test	p value
Sex				
Male	78.8	50.2-107.5		
Female	44.0	17.8-70.3	-2.677	0.102
Age				
< 65 years	78.7	55.5-101.8		
≥ 65 years	45.3	17.1-73.6	6.486	0.011
Tumor location				
Single lobe	40.1	20.3-59.9		
Multiple lobes	22.4	3.8-40.9		
Central area	71.9	46.7-97.2		
Others	123.9	86.2-161.6	10.664	0.014
Surgical approach				
Biopsy	44.4	28.1-60.7		
Craniotomy	72.0	41.1-102.9	0.067	0.796
Treatment protocol				
Chemotherapy	62.1	36.3-93.3		
Radiotherapy	62.1	57.1-121.3		
Radiochemotherapy	89.2	18.0-106.3		
No treatment	4.9	.00-10.3	14.727	0.002
Chemotherapy				
Yes	83.2	59.8-106.7		
No	26.0	0.00-54.6	5.245	0.022
Alkylating agents				
Yes	78.2	56.8-99.6		
No	41.1	12.3-69.8	7.295	0.007
Radiotherapy				
Yes	73.5	41.9-105.1		
No	48.5	24.9-72.2	2.283	0.131
Both alkylating agents and radiotherapy				
yes	67.5	46.8-88.2		
no	57.8	34.2-81.3	3.782	0.052
Ku80 expression				
Low	80.4	53.5-107.2		
High	55.3	29.6-88.7	4.377	0.036
MGMT expression				
Low	63.9	34.6-93.2		
High	59.7	34.3-85.1	0.142	0.706
Co-expression				
Ku80/MGMT(+)	66.4	37.2-95.7		
Ku80(+) or MGMT(+)	75.4	43.4-107.5		
Ku80/MGMT(-)	39.5	14.1-64.8	2.824	0.244

Tab. 8 Kaplan–Meier analyses for the correlation between variables and PFS.

Variables	PFS Mean	95% CI	log-rank test	*p* value
Sex				
Male	77.7	48.2-107.1		
Female	46.8	20.0-73.5	2.028	0.154
Age				
< 65 years	78.2	54.69-101.7		
≥ 65 years	45.4	16.2-74.4	5.902	0.015
Tumor location				
Single lobe	40.9	21.2-60.6		
Multiple lobes	20.9	1.6-40.3		
Central area	70.1	44.5-95.8		
Others	125.5	85.0-161.9	10.443	0.015
Surgical approach				
Biopsy	45.6	29.4-61.7		
Craniotomy	70.7	39.1-102.2	0.061	0.806
Treatment protocol				
Chemotherapy	64.7	35.9-93.4		
Radiotherapy	65.1	29.3-100.9		
Radiochemotherapy	89.9	58.0-121.9		
No treatment	3.0	0.0-8.1	13.294	0.004
Chemotherapy				
Yes	83.7	60.2-107.2		
No	25.0	0.0-54.3	4.606	0.032
Alkylating agents				
Yes	78.5	57.1-99.9		
No	40.3	10.9-69.6	6.845	0.009
Radiotherapy				
Yes	74.7	42.1-107.3		
No	47.8	23.7-71.9	2.039	0.153
Both alkylating agents and radiotherapy				
yes	67.2	46.1-88.3		
no	57.0	33.1-80.9	3.559	0.059
Ku80 expression				
Low	79.2	51.7-106.7		
High	58.6	32.8-84.4	3.987	0.046
MGMT expression				
Low	62.1	31.7-92.5		
High	61.2	36.2-86.3	0.002	0.964
Co-expression				
Ku80/MGMT(+)	64.5	36.7-92.3		
Ku80(+) or MGMT(+)	74.2	41.5-106.9		
Ku80/MGMT(-)	43.3	19.1-67.5	1.709	0.426

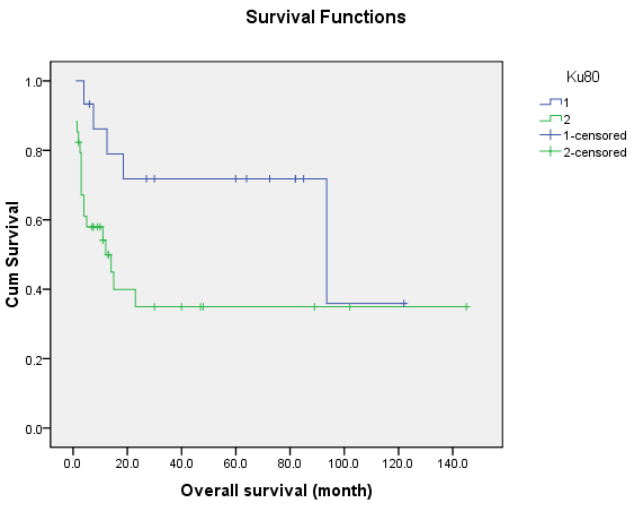

Fig. 9

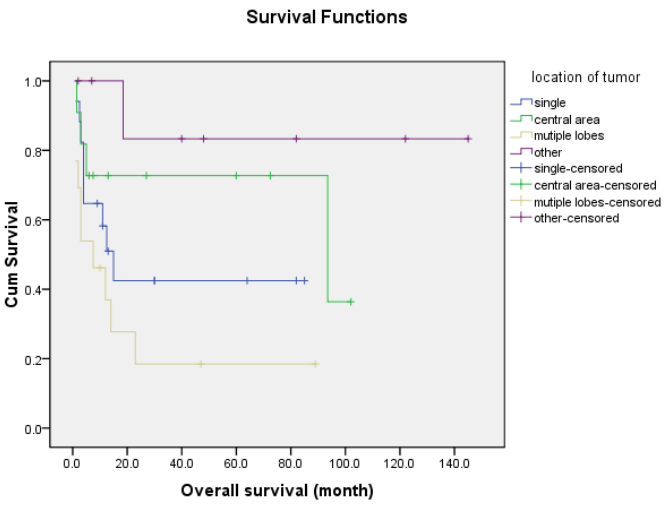

Fig. 10

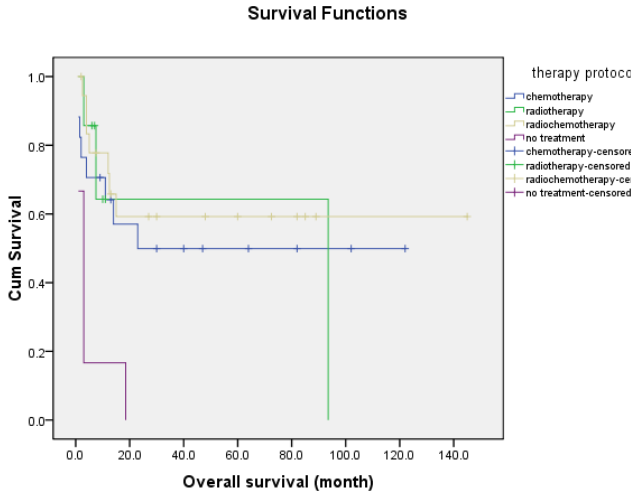

Fig. 11

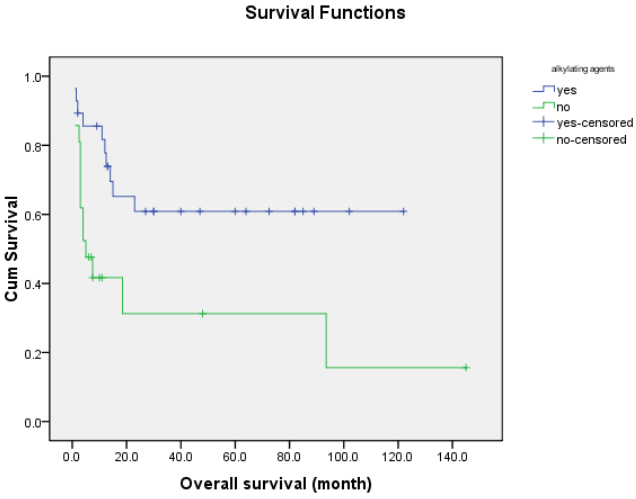

Fig. 12

Fig. 9~12 Kaplan-Meier survival curves of patients with PCNSL and its impact on overall survival were analyzed according to the expression level of Ku80 (Fig. 9,tumor location (Fig. 10), treatment protocol (Fig. 11), the use of alkylating agents (Fig. 12)

4. DISCUSSION

PCNSL has been paid more attention in recent years. Especially its unsatisfactory therapy and poor prognosis has been noticed, resulting in an increasing scientific awareness [3, 5, 7]. It is clear that its unsatisfactory therapy and poor prognosis are connected with the chemosensitivity and radiosensitivity [2, 26]. Special attention has been paid in recent years to factors related to the molecular biological characteristics of the tumor, in an attempt to predict and improve the prognosis. This study was aimed at detecting the expression of MGMT and Ku80 on PCNSL by IHC staining to evaluate the relationship between Ku80, MGMT expression levels and the clinical outcome, thus determine whether these immunophenotypes were prognostic markers in PCNSL. A second goal was to find out more about the general characteristics and prognosis of PCSNL.

4.1 Ku80 expression level in PCNSL and outcome, Ku80 may be a prognostic marker.

Despite many studies performed with established cell lines, little is known about Ku80 expression in lymphoma. The expression Ku80 in primary central nervous system lymphoma or DLBCL has not been published. The Ku70 showed relatively equal expression in different normal tissues, but Ku80 expression was somewhat more variable from tissue to tissue [63]. This study is the first to detect the expression Ku80 in primary central nervous system lymphoma tissue. The results of our exploratory study showed that a wide range expression of Ku80 was found among samples obtained from PCNSL patients. A considerable Ku80 expression level in PCNSL was found with mean expression rate 64.1%. A high expression of Ku80 correlated with a poor prognosis of PCNSL. The results of Ku80 expression by gender, tumor location showed no significant difference.

These result suggest significant difference between Ku80 expression (weak or high) and patients' age in our study, when patients were divided into two groups with separation younger/older than 65 years. Different statistical methods lead to different results, one of reasons is that this graded statistical method has its shortage

of loss of some information. Enlarging sample sizes and more layers graded could make up for its defect and reduce the loss of information. One also may explain that the difference of Ku80 expression between different age groups may be due to age-dependent changes in tumor. It is also reported that among the DSB repair proteins tested, the expression of Ku70 showed statistically significant age- dependent changes in human lymphocytes, but the expression of Ku80 didn't [64] . Whether Ku80 expression is age-dependent in PCNSL is still unknown.

The success of DSB repair in tumor cells with high expression of Ku80 is the major cause for radiotherapy failure, leading to prolonged tumor cell survival, and tumors with a low percentage of Ku80-positive cells tend to be radiosensitive. Ku80 may be a potential prognostic marker for the prediction of radiotherapy outcome. In our study, Kaplan-Meier analysis revealed that patients who had high Ku80 expression had significantly shorter MST than patients who had a low Ku80 expression ($P = 0.036$). This observation suggested that Ku80 expression might be of prognostic significance in the patients with PCNSL whose tumor cells manifest higher levels of Ku80 expression. However, the influence of Ku80 on survival in patients with and without radiotherapy, no statistical significance was found ($P > 0.05$). So, Ku80 may be a marker to prognosis, but can't predict the therapeutic effectiveness of radiotherapy, and of course can't work as an indicator to radiotherapy.

Although it is supported that Ku protein expression is correlated with radiation treatment outcome [53-55] [56], there are exceptions, some stated that Ku80 expression is no correlated with radiation sensitivity [65]. Although Ku80 levels were detected to correlate significantly to OS in our study, there was no significance found between radiotherapy and outcome. This observation supports Kasten-Pisula results that Ku80 expression is not correlated with radiation sensitivity. It may be one explanation that Ku80 play a role on the repair of DNA ends by nonhomologous end joining (NHEJ) cooperated with other compounds such as Ku70, DNA-PKCS, Xrcc4, DNA ligase IV and others.

A slight increase in cellular levels of Ku80 after irradiation in both human fibroblasts and lymphoblasts could be shown, while the expression of Ku70 remarkably increased [51]. This observation suggests that a radiation-resistant phenotype is determined by Ku70 expression. On the other hand, a remarkable increase of Ku80 expression, compared to Ku70 expression, occurred after irradiation [56]. The expression and functions Ku80 maybe vary widely in various tumors. Although the exact functions of Ku70 and Ku80 are not yet known, the induction of Ku80 expression by ionizing radiation is a key step in radiation resistance.

4.2 MGMT expression level in PCNSL

Most association analyses between MGMT and lymphoma were performed in DLBCL subgroup of systemic lymphoma. The MGMT status in other subgroup lymphoma is still unclear. The expression of MGMT protein tested by IHC is rarely reported. MGMT protein protects cells from toxicity of alkylating agents that frequently target the O6 position of guanine [66]. It is therefore assumed that the good prognosis of DLBCL with low MGMT expression was caused by a better response to alkylating agents such as cyclophosphamides. Esteller et al reported that MGMT promoter hypermethylation appears to be a useful marker for predicting survival in patients with DLBCL treated with multidrug regimens [67]. A significant difference of the prognosis was observed between DLBCL with and without silencing of the DNA repair enzyme MGMT in DLBCL [68]. Similar results were described by Al-Kuraya et al [66]. Recently, Lee and his co-workers concluded that aberrant promoter methylation of MGMT is an additional biological marker for an increased overall survival in patients with DLBCL[69].

However, the MGMT status in PCNSL and its correlation to clinical outcome is still unknown. MGMT methylation in one of three patients with PCNSL was reported by Gonzalez-Gomez [39]. Chu et al found promoter methylation of MGMT in PCNSL was correlated with the loss of MGMT protein expression. There is also evidence that MGMT methylation status may be an important predictor for the response to alkylating agents in patients with PCNSL [70].

In our study, the level of MGMT expression was detected by IHC method in 49 patients with PCNSL. 26 (53.1%) tumor samples demonstrated intermediate and high MGMT expression respectively. Consistent with our results, Chu, et al observed a similar rate of MGMT expression in PCNSL with methylation-specific PCR method [70]. There are no significant differences in MGMT expression level with patients' gender, age, and locations of tumors. No significant difference on survival between patients who had high MGMT expression and patients with low MGMT expression was observed by Kaplan-Meier method (P =0.706, log-rank test). So, in the present study, MGMT protein expression can not be considered a predictor to chemotherapeutic response and prognosis in patients with PCNSL.

We showed in our study that patients treated with alkylating agents had a significantly longer MST (P =0.007). This supports the conclusion that alkylating agents play an important role in chemotherapy outcomes in patients with PCNSL. There are three possible explanations to ths surprising result: a) This phenomenon is caused by an error of analysis techniques, it may be one way to choose adequate method or combine with other methods such as methylation specific PCR for preferable assessing the MGMT status. b) Sample size is not enough, further analysis with larger number of cases is necessary to clarify the utility of MGMT immunohistochemistry as a predictor of prognosis of PCNSL. c) Given alkylating agents prescribed as part of multidrug regimens, alkylating agents play a role on tumor cells through other mechanisms [71], for example, its synergy with other chemotherapy drugs, bi-functional alkylating activity [72], or its bimodal mechanism of antitumor action with cytotoxic and immunomodulatory effects [73]. Marchesi et al. reviewed that the biological effects of triazene compounds depends on at least three DNA repair systems: a) O6-alkylguanine-DNA-alkyltransferase, called also methyl-guanine methyl-transferase (MGMT) b) mismatch repair (MMR), and c) base excision repair (BER) [71].

We conclude that alkylating agents play an important role in treatment of patients with PCNSL, but the expression level of MGMT is not a predictor of a

successful alkylating treatment. To proof this hypothesis further studies and larger case series might be necessary.

4.3 Ku80 and MGMT level in primary CNSL and secondary CNSL

More and more clinical trials and studies are no longer only based on the histological diagnoses, but also take into consideration the molecular markers such as the MGMT promoter methylation. It is well known that biomarkers expressed in CNS DLBCL are not of the same prognostic significance as in non- CNS DLBCL. Although, the vast majority of primary CNSL and secondary CNSL are DLBCL according to histological classification [2]. The role of Ku80, MGMT and the relevance for the different clinical characteristics of primary and secondary CSNL is unclear. There is no recent literature regarding this issue.

Our attention was therefore directed on detecting the expression of Ku80 and MGMT in primary CNSL and secondary CNSL. To keep it short, we could not find significant difference for both Ku80 and MGMT in primary CNSL compared with secondary CNSL. We can only speculated, that the expression of Ku80 and MGMT in CNSL may be determined by his DLBCL histological subtype.

4.4 Ku80 and MGMT co-expression in PCNSL

High Ku80 expression was associated with reduced survival in Kaplan-Meier analysis. There was no significant impact of MGMT expression on prognosis. A strong trend was found toward poor prognosis in MGMT/Ku80 positive as compared with MGMT/Ku80 negative cases. The difference, however, did not reach statistical significance ($P = 0.244$). Furthermore, we found that expressions of Ku80 and MGMT are statistically correlated ($p=0.029$), which indicate that the expression of Ku80 and MGMT may be related.

Both Ku80 and MGMT are DNA repair genes; they play an important role on protecting tumor cells against DNA injuries caused by chemotherapy and radiotherapy. The Ku80, as one of the subunit of Ku complex, plays a key role in multiple nuclear processes, for example, the repair of DNA ends by nonhomologous end joining (NHEJ), chromosome maintenance, transcription regulation and VJ recombination

[43-45], whereas the MGMT-mediated repair process is unique and differs from other DNA repair pathways as MGMT is not part of a repair complex but acts alone [27]. There may be some unknown molecular biological mechanism making the regulation of their co- expression in human tumor cells.

4.5 Prognostic factors in PCNSL

The biological mechanism involved in pathogenesis of PCNSL is complex but deserve further study. It is possible and important to search appropriate prognostic factors not only for division of patients with PCNSL into specific risk groups for identification and assessment of appropriate therapies, but also for predicting the survival. The traditional markers of prognosis in non-Hodgkin's lymphoma, which form the International Lymphoma Study Group classification[74]—namely, performance status, LDH levels, and disease stage—may be not of prognostic markers in PCNSL.

Age and performance status (PS) are the only two universally accepted prognostic factors [23, 75, 76]; several other potential prognostic parameters such as histotype, duration of symptoms, subtentorial localization, and bilateral brain involvement were proposed but failed to be confirmed in subsequent studies. Recently, the IELSG prognostic score, which consisted of 5 parameters: age, KPS, serum LDH level, CSF protein concentration and involvement of deep regions of the brain, are correlated with a negative prognosis in PCNSL [22].

In our survival analysis, the expression of Ku80 was considered as an important predictor, whereas MGMT was no statistically significant, although it was discovered that MGMT is an independent prognostic marker in DLBCL [66]. Therefore usefulness of MGMT immunohistochemstry for the prediction of chemosensitivity of PCNSL is limited. It is not surprising that age is a prognostic factor as expected in this study. In addition to Ku80 and age, this study reported an independent prognostic role of the involvement of location of the tumor in PCNSL patients. In fact, the involvement of periventricular regions, basal ganglia, brainstem, and/or cerebellum was associated with a poor prognosis. Treatment protocol and

alkylating agents played a prognostic role independent of age and PS with statistically significance. Nevertheless, it is plausible that age and PS have influenced patient's selection to receive more or less aggressive therapy such as chemotherapy combinations and doses. It is concluded that patients' age, tumor location, treatment protocol and the use alkylating agents are factors to predict prognosis in patients with PCNSL.

4.6 MGMT analysis techniques

It is noteworthy that most MGMT analyses in the literature were performed retrospectively using a variety of different techniques. Large intergroup prospective studies utilizing standardized techniques are therefore necessary to validate such findings. A number of methods have been described to assess the status of the MGMT: a MSP assay for methylation status of the MGMT gene promoter region, reverse-transcription (RT)–PCR for MGMT mRNA expression, IHC and a quantitative protein expression assay using western blotting and many others. The best technique for evaluating MGMT status is still a matter of debate.

More recently, the use of polymerase chain reaction (PCR) allowed the analysis of small populations of cells from tissue fragments or cerebrospinal fluid. There is growing acceptance that MGMT promoter methylation assessment by methylation specific PCR (MSP) is more accurate than detection by immunohistochemistry (IHC) because MGMT promoter methylation does not correlate well with MGMT protein expression [77]. Most authors recommend assessment of MGMT promoter methylation status using the more specific MSP technique which requires frozen tumor sections for optimal results.

However, MSP is a relatively complicated and time-consuming method, not available generally in the local hospital. In addition, the formalin fixation and paraffin embedding of tumor tissues deteriorates the DNA quality in the tissue, which may lead to failure of amplification by MSP, particularly in small samples (e.g., stereotactic biopsies) [78]. Requiring fresh vital tissues or fresh frozen specimens limits the applicability of this method in routine practice.

Some authors prefer to IHC technique for evaluating MGMT status because promoter methylation status does not always reflect protein expression. There are several potential advantages of IHC compared with MSP. IHC, as a routinely reliable method in diagnostic histopathology, is commonly available in most laboratories. Furthermore, IHC is less expensive than MSP and works on formalin-fixed and paraffin-embedded tissues. Several studies have reported there is a significant correlation between immunohistochemically assessed MGMT expression and patient outcome in glioma [78-81]. Some more recent studies reported that pre- therapy analysis of MGMT protein expression in malignant gliomas may help to identify patients in whom tumors are resistant to TMZ. Anda et al reported strong immunohistochemical MGMT staining in 18 patients with glioblastomas may show more chemoresistance to alkylating drugs [79]. Chinot et al found patients that MGMT expression is correlated with response to TMZ in 29 glioblastoma [80]. Similar results were reported for pediatric patients with malignant gliomas [81]. So there is great interest in the clinical use of MGMT immune- staining. According to their respective advantages and shortcomings, it is essential to choose the adequate method or combine these techniques for preferable assessing the MGMT status. For these reasons, we preferred IHC to evaluate the expression of MGMT in our study.

5. CONCLUSIONS

The expression of Ku80 and MGMT was observed in the majority of PCNSLs, and without statistical difference compared with their expressions in secondary CNS lymphoma. It is the first time to detect the expression of Ku80 proteins in PCNSL with IHC method. Ku80 was predictive for survival in this study. Immunohistochemical detection of MGMT in PCNSL does not correlate with overall survival, and cannot be used as a prognostic factor. In addition to Ku80 expression, other clinical variables including tumor location, treatment protocol and alkylating agents are correlated significantly with overall survival. The role of these variables and the clinical relevance deserve to be assessed in further studies.

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7. SUMMARY

The primary central nervous system lymphoma (PCSNL), as one of the uncommon extranodal lymphomas, has been recently paid more attention especially for its increasing incidence, unsatisfactory therapy and poor prognosis. MGMT is one of the most important factors determining drug resistance while Ku80 determining radiosensitivity, the expression of MGMT and Ku80 in PCNSL remains unclear. The aim of our study was to detect the expression of MGMT and Ku80 on PCNSL by IHC staining and to evaluate the relationship between Ku80, MGMT expression level and clinical outcomes, thus determine whether these immunophenotypes were prognostic factors in PCNSL.

49 patients with PCNSL were included in this retrospective study. The expression of Ku80 and MGMT in tumor samples was determined by immunohistochemistry using the Ku80 and MGMT monoclonal mouse antihuman antibody. One thousand neoplastic cells per specimen were counted. On the basis of the percentages of positive cells in the tumors, these tumors were defined as low Ku80 expression or low MGMT expression when there are no or fewer than 50% positive cells, and high expression when positive rate was more than 50%. The expression levels were than compared to the clinical data and statistical analyzed.

The mean expression level of Ku80 and MGMT in 49 PCNSL were 64.1±24.5 and 51.3±29.5 respectively. A correlation was found between these two proteins expressions ($r=0.311$, $P=0.029$). A significant difference in Ku80 expression could be found between age<65 years group and age≥65 years group ($P=0.006$), Differences in Ku80 and MGMT expression between primary and secondary CNS lymphomas did not reach significance ($P>0.05$). Kaplan-Meier analysis revealed that patients who showed a high Ku80 expression had a significantly shorter median survival time (MST) than patients who had low Ku80 expression (55.3 months vs. 80.4 months; $P=0.036$). This could not be observed for the MGMT expression. Patients' age, tumor location, treatment protocol, alkylating agents were significantly

related with prognosis in PCNSL ($P<0.05$).

The results of this study show that the expression of Ku80 and MGMT can be found in the majority of PCNSLs, although without statistically difference compared with their expressions in secondary CNS lymphoma. It is the first time to detect the expression of Ku80 proteins in PCNSL. Ku80 expression was a positive predictor for survival in this study. Immunohistochemical detection of MGMT does not correlate with overall survival, and cannot be used as a prognostic factor. With Ku80 we can add another predictor to evaluate the prognosis in patients with PCSNL and confirm the relevance of patient age and tumor location at the time of diagnosis in the prognosis of PCSNL.

8. Abbreviation List

ALL	Acute lymphocytic leukemia
Ara-C	Cytarabine
BER	Base excision repair
CHOP	Cyclophosphamide, hydroxydaunorubicin (Adriamycin), Oncovin (vincristine), and prednisone/prednisolone
CLL	Chronic lymphocytic leukemia
CNS	Central nervous system
CP	Cyclophosphamide
CR	Complete remission
CSF	Cerebrospinal fluid
DLBCL	Diffuse large B-cell lymphoma
DNA-PKcs	DNA-PK catalytic subunit
DNA-PK	DNA-dependent protein kinase
DSB	DNA double-strand breaks
GBM	Glioblastoma multiforme
HCL	Hydrogen chloride
IELSG	The International Extranodal Lymphoma Study Group
IFO	Ifosphamide
IHC	Immunohistochemistry
KPS	Karnofsky performance status
LDH	Lactate dehydrogenase
MGMT	O6-MethylguanineDNAmethyltransferase
MMR	Mismatch repair
MSP	Methylation specific PCR
MST	Median survival time
MTX	Methotrexate

NaCl	Sodium Chloride
NHEJ	Nonhomologous end joining
NOVEP	Mitoxantrone, Etoposide and Prednisolone
OS	Overall survival
PBS	Phosphate buffered saline
PCNSL	Primary central nervous system lymphoma
PFS	Progression-free survival
PR	Partial remission
PS	Performance status
REAL	Revised European-American Lymphoma
RT–PCR	Reverse-transcription polymerase chain reaction
TBS	Tris-buffered saline
TMZ	Temozolomide
WBRT	Whole-brain radiotherapy
WHO	World Health Organization

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